ANALYTICAL CHARACTERIZATION OF BIOLOGICAL PRODUCTS

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by

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DEDICATED TO MAA, PAPA, SACHIN,

AND MY DEAREST FRIEND JAIN

CERTIFICATE

This is to certify that the thesis entitled **"ANALYTICAL CHARACTERIZATION OF BIOLOGICAL PRODUCTS"** being submitted by **Mr. Ramesh Kumar** to the Indian Institute of Technology Delhi for the award of the degree of **"Doctor of Philosophy"** is a record of the original bonafide research work carried out by him under my guidance and supervision in conformity with rules and regulation of the Indian Institute of Technology Delhi, India. The results contained in this thesis have not been submitted in part or in full to any other University or Institute for the award of any degree or diploma.

I certify that he has pursued the prescribed course of research.

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"If you want a happy ending, that depends, of course, on where you stop your story"

Orson Welles

RAMESH KUMAR

Abstract

The term "biological products" refers to the therapeutic products that are manufactured by living organisms. Currently these biologicals, also called biotherapeutics, dominate the pharmaceutical market. However, these are sensitive to perturbations in their immediate environment which can affect their potency, efficacy, and at times, even safety. Analytical characterization of these biological products is thus paramount, both for the designing of new drugs and for analyzing those already available in the market. Depending upon the information that requires detailing, several analytical tools and techniques can be employed for in-depth characterization of the molecule of interest. These include enzyme-linked immunosorbent assay (ELISA), surface plasmon resonance (SPR), high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), mass spectrometry (MS), dynamic light scattering (DLS), and cryo-electron microscopy (cryo-EM).

The current work demonstrates how we can utilize some of the aforementioned technologies for in-depth and fast characterization of a variety of biological products, including monoclonal antibodies (mAbs) and their biosimilars, host cell proteins (HCPs), antivenoms, and virus-like particles (VLPs).

The first objective involves utilization of different cyclodextrins (CDs) for the improved detection of mAb charge variants. Charge heterogeneity is a critical quality attribute (CQA) for mAb-based therapeutics. Its characterization involves identification of charge variant species in the final formulation. Capillary zone electrophoresis (CZE) is a valuable tool to assess charge heterogeneity, but typically offers incomplete separation of the different product’s isoforms. CD-mediated chiral CE is a well-established electromigration method. We demonstrate how we can use CDs as modulators for CZE-based separation of mAb charge variants (biosimilars of Rituximab and Trastuzumab). Both the negatively charged carboxymethyl-beta-cyclodextrin (CMβCD) and uncharged (2-Hydroxypropyl)-beta-cyclodextrin (HPβCD) have been studied. The effect of CD concentration has been found to impact the extent of modulation of resolution of charge variants. Addition of 10 mM CMβCD not only improved resolution but also resulted in separation of two additional basic and one additional acidic species for Rituximab. Similarly, besides improvement in the resolution, one additional basic variant was observed with 2.5 mM CMβCD with Trastuzumab. No improvement in resolution was, however, observed with HPβCD, suggesting the selective utility of CDs for analysis of mAb charge variants.

The second objective of this project was to establish a method to improve CE throughput for the analysis of mAbs. The complexity of biotherapeutic products implies an ever-increasing list of product quality attributes that need to be monitored and characterized. In addition, the growing interest in implementing process analytical technology (PAT) in biopharmaceutical production has further increased the testing burden, together with the need for rapid testing that can facilitate real-time or near-real-time decision-making. CE has made a place in biopharmaceutical analysis but is regarded as a low-throughput method, with the instrument dead time comprising 80 % of the total analysis time. In this study, the dead time of CE was utilized to analyze 3 mAb samples in a single-CE run. This approach resulted in an up to 77 % reduction in the total analysis time and increased the productivity by up to 300 %, compared to traditional single CE-ultraviolet (UV) runs, without compromising the resolution or the relative peak areas. Additionally, good method reproducibility was observed. The compatibility of the method has been demonstrated with protein A eluate and cation exchange chromatography fractions.

The third objective was the establishment of an offline multidimensional method using liquid chromatography (LC) in conjunction with CE and tandem mass spectrometry (MS/MS). LC is the most frequently used technique in the industry for characterization of biotherapeutics. It does, however, have significant drawbacks, such as a reduced sensitivity to hydrophilic or small peptides. CE is an emerging tool and offers results that are complementary to those of LC but has limited loading capacity which limits its sensitivity. Thus, a combined platform that harnesses the power of both chromatography and electrophoresis has been developed and tested for peptide mapping analysis of mAbs. For this, the trypsin digested mAb was first fractionated on LC, and each fraction was analyzed by CE-MS in an offline manner. This method, called LC-CE-MS, identified more peptides of varying properties, and greatly improved the sequence coverage and post-translational modifications (PTMs) analyses compared to the traditional one-dimensional (1D) LC and CE methods. In fact, the system performed better than even the combined datasets of LC-MS and CE-MS. Hence, this two-dimensional (2D) LC-CE-MS method was proposed as an addition to the analytical tool box for primary characterization of mAbs.

The fourth goal was to use the previously established LC-CE-MS method to identify host cell proteins (HCPs) in the culture supernatant of a Chinese Hamster Ovary (CHO) cell line that is commonly used for production of mAbs. Although many of these HCPs may be identified

using LC-MS or CE-MS alone, it can be challenging to identify all of them due to the complexity of the material, and the limited peak capacity of the system. Accordingly, LC-CE-MS identified more than twice the number of HCPs compared to LC-MS and CE-MS alone. Furthermore, a greater abundance of peptidases was also detected with LC-CE-MS. The method was found to be better for CHO secretome analysis than the conventional 1D methods.

The fifth goal was to analyze the interactions between an antivenom therapeutics candidate and snake venoms. A functional characterization of recombinant lethal toxin neutralizing factor (rLTNF) against cobra venom (CV) and Russell's viper venom (RVV) was performed using LC-MS. The LTNF is a peptide-based antivenom candidate which is known to be active against a number of snake venoms. In this study we investigated the interactions of this rLTNF peptide with cobra and Russell’s viper venoms. Besides the monomeric peptide, the potential of rLTNF oligomers was also investigated. The rLTNF oligomers were found to severely affect the chromatographic profiles for both CV and RVV, indicating their potential to be used as antivenom therapeutics.

The creation of an LC-ESI-MS-based approach for the detection of conjugated Flock house virus (FHV) virus-like particles (VLPs) was the final goal of this study. Conjugated peptides have been used to improve the specificity of therapeutic VLPs by guiding them to their intended targets. However, deciphering conjugation is analytically challenging. In this instance, based on the masses, and retention times changes, we utilized an LC-ESI-MS-based method to distinguish between conjugated and non-conjugated VLPs. Peptide conjugation resulted in significant changes and shifts in the chromatogram and MS spectra. To the best of our knowledge, this is the first report on the use of ESI-MS for such analysis.

Together, we demonstrated the use of different analytical tools for improved characterization of different classes of biopharmaceuticals. These aforementioned methods could constitute useful additions to the analytical toolbox for better and faster characterization of biotherapeutics.

सार

शब्द "जैविक उत्पाद" उन चिकित्सीय उत्पादों को संदर्भित करता है जो जीवित जीवों द्वारा निर्मित होते हैं। वर्तमान में ये जैविक, जिन्हें बायोथेराप्यूटिक्स भी कहा जाता है, दवा बाजार पर हावी हैं। हालाँकि, ये अपने तात्कालिक वातावरण में गड़बड़ी के प्रति संवेदनशील होते हैं जो उनकी क्षमता, प्रभावकारिता और कभी-कभी सुरक्षा को भी प्रभावित कर सकते हैं। इस प्रकार नई दवाओं के डिजाइन और बाजार में पहले से उपलब्ध दवाओं के विश्लेषण के लिए इन जैविक उत्पादों का विश्लेषणात्मक लक्षण वर्णन सर्वोपरि है। उस जानकारी के आधार पर जिसके लिए विवरण की आवश्यकता होती है, रुचि के अणु के गहन लक्षण वर्णन के लिए कई विश्लेषणात्मक उपकरण और तकनीकों को नियोजित किया जा सकता है। इनमें एंजाइम-लिंक्ड इम्युनोसॉरबेंट परख (एलिसा), सतह प्लास्मोन अनुनाद (एसपीआर), उच्च-प्रदर्शन तरल क्रोमैटोग्राफी (एचपीएलसी), केशिका वैद्युतकणसंचलन (सीई), मास स्पेक्ट्रोमेट्री (एमएस), गतिशील प्रकाश बिखरने (डीएलएस) और क्रायो-इलेक्ट्रॉन माइक्रोस्कोपी (क्रायो-ईएम) शामिल हैं।

वर्तमान कार्य दर्शाता है कि हम मोनोक्लोनल एंटीबॉडी (एमएबीएस) और उनके बायोसिमिलर, मेजबान सेल प्रोटीन (एचसीपी), विषरोधी, और वायरस जैसे कण (वीएलपी) सहित विभिन्न जैविक उत्पादों के गहन और तेज़ लक्षण वर्णन के लिए उपरोक्त कुछ तकनीकों का उपयोग कैसे कर सकते हैं।

पहले उद्देश्य में एमएबी चार्ज वेरिएंट की बेहतर पहचान के लिए विभिन्न साइक्लोडेक्सट्रिन (सीडी) का उपयोग शामिल है। एमएबी-आधारित चिकित्सा विज्ञान के लिए चार्ज विविधता एक महत्वपूर्ण गुणवत्ता विशेषता (CQA) है। इसके लक्षण वर्णन में अंतिम फॉर्मूलेशन में चार्ज वैरिएंट प्रजातियों की पहचान शामिल है। केशिका क्षेत्र वैद्युतकणसंचलन (सीजेडई) चार्ज विविधता का आकलन करने के लिए एक मूल्यवान उपकरण है, लेकिन आम तौर पर विभिन्न उत्पाद के आइसोफॉर्म का अधूरा पृथक्करण प्रदान करता है। सीडी-मध्यस्थता चिरल सीई एक अच्छी तरह से स्थापित इलेक्ट्रोमाइग्रेशन विधि है। हम प्रदर्शित करते हैं कि हम एमएबी चार्ज वेरिएंट (रिटक्सिमैब और ट्रैस्टुज़ुमैब के बायोसिमिलर) के सीजेडई-आधारित पृथक्करण के लिए मॉड्यूलेटर के रूप में सीडी का उपयोग कैसे कर सकते हैं। नकारात्मक रूप से चार्ज किए गए कार्बोक्सिमिथाइल-बीटा-साइक्लोडेक्सट्रिन (सीएमβसीडी) और अनावेशित (2-हाइड्रॉक्सीप्रोपाइल)-बीटा-साइक्लोडेक्सट्रिन (एचपीβसीडी) दोनों का अध्ययन किया गया है। सीडी एकाग्रता का प्रभाव चार्ज वेरिएंट के रिज़ॉल्यूशन के मॉड्यूलेशन की सीमा पर प्रभाव डालता पाया गया है। 10 एमएम सीएमβसीडी को जोड़ने से न केवल रिज़ॉल्यूशन में सुधार हुआ बल्कि रिटक्सिमैब के लिए दो अतिरिक्त क्षारीय और एक अतिरिक्त अम्लीय प्रजाति अलग हो गई। इसी तरह,

रिज़ॉल्यूशन में सुधार के अलावा, 2.5 एमएम सीएमβसीडी के साथ ट्रैस्टुज़ुमैब के साथ एक अतिरिक्त क्षारीय संस्करण देखा गया। हालाँकि, एचपीβसीडी के साथ रिज़ॉल्यूशन में कोई सुधार नहीं देखा गया, जो एमएबी चार्ज वेरिएंट के विश्लेषण के लिए सीडी की चयनात्मक प्रकृति उपयोगिता का सुझाव देता है।

इस परियोजना का दूसरा उद्देश्य एमएबीएस के विश्लेषण के लिए सीई थ्रूपुट में सुधार करने के लिए एक विधि स्थापित करना था। बायोथेराप्यूटिक उत्पादों की जटिलता का तात्पर्य उत्पाद की गुणवत्ता विशेषताओं की लगातार बढ़ती सूची से है, जिनकी निगरानी और विशेषता की आवश्यकता होती है। इसके अलावा, बायोफार्मास्युटिकल उत्पादन में प्रक्रिया विश्लेषणात्मक प्रौद्योगिकी (पीएटी) को लागू करने में बढ़ती रुचि ने परीक्षण के बोझ को और बढ़ा दिया है, साथ ही तेजी से परीक्षण की आवश्यकता भी बढ़ गई है जो वास्तविक समय या लगभग वास्तविक समय में निर्णय लेने की सुविधा प्रदान कर सकती है। सीई ने बायोफार्मास्युटिकल विश्लेषण में एक जगह बना ली है, लेकिन इसे कम-थ्रूपुट विधि के रूप में माना जाता है, जिसमें उपकरण का मृत समय कुल विश्लेषण समय का 80% होता है। इस अध्ययन में, सीई के मृत समय का उपयोग एकल- सीई रन में 3 एमएबीएस नमूनों का विश्लेषण करने के लिए किया गया था। इस दृष्टिकोण के परिणामस्वरूप कुल विश्लेषण समय में 77% तक की कमी आई और रिज़ॉल्यूशन या सापेक्ष चरम क्षेत्रों से समझौता किए बिना पारंपरिक एकल सीई-पराबैंगनी (यूवी) रन की तुलना में उत्पादकता में 300% तक की वृद्धि हुई। इसके अतिरिक्त, अच्छी विधि प्रतिलिपि प्रस्तुत करने योग्यता देखी गई। विधि की अनुकूलता प्रोटीन ए इलुएट और कटियन एक्सचेंज क्रोमैटोग्राफी अंशों के साथ प्रदर्शित की गई है।

तीसरा उद्देश्य सीई और टेंडेम मास स्पेक्ट्रोमेट्री (एमएस/एमएस) के संयोजन में तरल क्रोमैटोग्राफी (एलसी) का उपयोग करके एक ऑफ़लाइन बहुआयामी विधि की स्थापना करना था। बायोथेरेप्यूटिक्स के लक्षण वर्णन के लिए एलसी उद्योग में सबसे अधिक इस्तेमाल की जाने वाली तकनीक है। हालाँकि, इसमें महत्वपूर्ण कमियाँ हैं, जैसे हाइड्रोफिलिक या छोटे पेप्टाइड्स के प्रति कम संवेदनशीलता। सीई एक उभरता हुआ उपकरण है और ऐसे परिणाम प्रदान करता है जो एलसी के पूरक हैं लेकिन इसकी लोडिंग क्षमता सीमित है जो संवेदनशीलता को सीमित करती है। इस प्रकार, एक संयुक्त मंच जो क्रोमैटोग्राफी और इलेक्ट्रोफोरेसिस दोनों की शक्ति का उपयोग करता है, उसे एमएबीएस के पेप्टाइड मैपिंग विश्लेषण के लिए विकसित और परीक्षण किया गया। इसके लिए, ट्रिप्सिन पचाए गए एमएबी को पहले एलसी पर विभाजित किया गया, और प्रत्येक अंश का ऑफ़लाइन तरीके से सीई-एमएस द्वारा विश्लेषण किया गया। एलसी-सीई-एमएस नामक इस विधि ने अलग-अलग गुणों के अधिक पेप्टाइड्स

की पहचान की, और पारंपरिक एक आयामी (1डी) एलसी और सीई तरीकों की तुलना में अनुक्रम कवरेज और पोस्ट-ट्रांसलेशनल संशोधनों (पीटीएम) विश्लेषणों में काफी सुधार किया। वास्तव में, सिस्टम ने एलसी-एमएस और सीई-एमएस के संयुक्त डेटासेट से भी बेहतर प्रदर्शन किया। इसलिए, इस दो आयामी (2डी) एलसी-सीई-एमएस विधि को एमएबीएस के प्राथमिक लक्षण वर्णन के लिए विश्लेषणात्मक टूल बॉक्स के अतिरिक्त के रूप में प्रस्तावित किया गया।

चौथा लक्ष्य चीनी हैम्स्टर ओवरी (सीएचओ) सेल लाइन, जो आमतौर पर एमएबी के उत्पादन के लिए उपयोग की जाती है, के कल्चर सुपरनैटेंट में मेजबान सेल प्रोटीन (एचसीपी) की पहचान करने के लिए पहले से स्थापित एलसी-सीई-एमएस विधि का उपयोग करना था । हालाँकि इनमें से कई एचसीपी को अकेले एलसी-एमएस या सीई-एमएस का उपयोग करके पहचाना जा सकता है, लेकिन सामग्री की जटिलता और सिस्टम की सीमित चरम क्षमता के कारण उन सभी की पहचान करना चुनौतीपूर्ण हो सकता है। तदनुसार, एलसी-सीई-एमएस ने अकेले एलसी-एमएस और सीई-एमएस की तुलना में एचसीपी की संख्या दोगुनी से अधिक की पहचान की। इसके अलावा, एलसी-सीई-एमएस के साथ पेप्टिडेस की अधिक मात्रा भी पाई गई। यह विधि पारंपरिक 1डी विधियों की तुलना में सीएचओ के स्रावित अणुओं के विश्लेषण के लिए बेहतर पाई गई।

पांचवां लक्ष्य एक एंटीवेनम चिकित्सीय उम्मीदवार और सांप के जहर के बीच परस्पर प्रभाव का विश्लेषण करना था। कोबरा जहर (सीवी) और रसेल के वाइपर जहर (आरवीवी) के खिलाफ पुनः संयोजक घातक विष निष्क्रिय करने वाले कारक (आरएलटीएनएफ) का एक कार्यात्मक लक्षण वर्णन एलसी-एमएस का उपयोग करके किया गया था। एलटीएनएफ एक पेप्टाइड-आधारित एंटीवेनम उम्मीदवार है जिसे कई सांपों के जहर के खिलाफ सक्रिय माना जाता है। वर्तमान अध्ययन में हमने कोबरा और रसेल वाइपर के जहर के साथ इस आरएलटीएनएफ पेप्टाइड के परस्पर प्रभाव की जांच की गई। मोनोमेरिक पेप्टाइड के अलावा, आरएलटीएनएफ ऑलिगोमर्स की क्षमता की भी जांच की गई। आरएलटीएनएफ ऑलिगोमर्स सीवी और आरवीवी दोनों के लिए क्रोमैटोग्राफिक प्रोफाइल को गंभीर रूप से प्रभावित करते पाए गए, जो एंटीवेनम चिकित्सीय के रूप में उपयोग किए जाने की उनकी क्षमता को दर्शाता है।

संयुग्मित फ्लॉक हाउस वायरस (एफएचवी) वायरस जैसे कणों (वीएलपी) का पता लगाने के लिए एलसी-ईएसआई-एमएस-आधारित दृष्टिकोण का निर्माण इस अध्ययन का अंतिम लक्ष्य था। संयुग्मित पेप्टाइड्स का उपयोग चिकित्सीय वीएलपी को उनके इच्छित लक्ष्यों तक निर्देशित करके उनकी विशिष्टता में सुधार करने के लिए किया गया है। हालाँकि, संयुग्मन को समझना विश्लेषणात्मक रूप से

चुनौतीपूर्ण है। इस उदाहरण में, द्रव्यमान और अवधारण समय में परिवर्तन के आधार पर, हमने संयुग्मित और गैर-संयुग्मित वीएलपी के बीच अंतर करने के लिए एलसी-ईएसआई-एमएस-आधारित पद्धति का उपयोग किया है। पेप्टाइड संयुग्मन के परिणामस्वरूप क्रोमैटोग्राम और एमएस स्पेक्ट्रा में महत्वपूर्ण परिवर्तन और बदलाव हुए। हमारी सर्वोत्तम जानकारी के अनुसार, इस तरह के विश्लेषण के लिए ईएसआई-एमएस के उपयोग पर यह पहला प्रतिवेदन है।

कुल मिलाकर, हमने बायोफार्मास्यूटिकल्स के विभिन्न वर्गों के बेहतर लक्षण वर्णन के लिए विभिन्न विश्लेषणात्मक उपकरणों के उपयोग का प्रदर्शन किया। ये उपरोक्त विधियाँ बायोथेराप्यूटिक्स के बेहतर और तेज़ लक्षण वर्णन के लिए विश्लेषणात्मक टूलबॉक्स में उपयोगी परिवर्धन का गठन कर सकती हैं।

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°C	Degree Celsius
1D	One Dimensional
2D	Two-Dimensional
ABC	Ammonium Bicarbonate
ACN	Acetonitrile
BGE	Background Electrolyte
CQA	Critical Quality Attribute
CZE	Capillary Zone Electrophoresis
Da	Dalton
DAD	Diode Array Detector
DTT	Dithiothreitol
ELISA	Enzyme-Linked Immunosorbent Assay
ESI	Electrospray Ionization
FA	Formic acid
FDR	False Discovery Rate
Gdn-HCl	Guanidine Hydrochloride
GO	Gene Ontology
HC	Heavy Chain
HCD	Host cell Deoxy-ribonucleic acid
HCP	Host Cell Protein
hr	Hour
IAM	Iodoacetamide

ICH	International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use
IDA	Information Dependent Acquisition
IgG	Immunoglobulin G
IMGT	The International ImMunoGeneTics Information system
LC	Light Chain
m/z	Mass-to-Charge ratio
mAb	Monoclonal Antibody
MAM	Multi-Attribute Method
min	Minute(s)
MS	Mass Spectrometry
MWCO	Molecular Weight Cut-Off
nm	Nanometer
NTD	Neglected tropical disease
PAGE	Polyacrylamide gel electrophoresis
PAT	Process analytical technology
pI	Isoelectric Point
ppm	Parts Per Million
PTM	Post Translational Modification
Q-TOF	Quadrupole Time-of-Flight
RPLC	Reversed Phase Liquid Chromatography
RT	Retention Time
s	Second
SBE	Snakebite envenoming

SCX	Strong Cation Exchange
SDS	Sodium dodecyl sulphate
TIC	Total Ion Chromatogram
TIC	Total Ion Chromatogram
TOF	Time-of-Flight
UV	Ultraviolet
V_{cap}	Capillary Voltage
V_{frag}	Fragmentor Voltage
VLP	Virus Like Particle
WHO	World Health Organisation
XIC	Extracted Ion Chromatogram
μL	Microliter